Inhibition of Calcium Dependent Protein Kinase 1 (CDPK1) by Pyrazolopyrimidine Analogs Decreases Establishment and Reoccurrence of Central Nervous System Disease by Toxoplasma gondii


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Supporting Information

ABSTRACT: Calcium dependent protein kinase 1 (CDPK1) is an essential enzyme in the opportunistic pathogen Toxoplasma gondii. CDPK1 controls multiple processes that are critical to the intracellular replicative cycle of T. gondii including secretion of adhesins, motility, invasion, and egress. Remarkably, CDPK1 contains a small glycine gatekeeper residue in the ATP binding pocket making it sensitive to ATP-competitive inhibitors with bulky substituents that complement this expanded binding pocket. Here we explored structure−activity relationships of a series of pyrazolopyrimidine inhibitors of CDPK1 with the goal of increasing selectivity over host enzymes, improving antiparasite potency, and improving metabolic stability. The resulting lead compound 24 exhibited excellent enzyme inhibition and selectivity for CDPK1 and potently inhibited parasite growth in vitro. Compound 24 was also effective at treating acute toxoplasmosis in the mouse, reducing dissemination to the central nervous system, and decreasing reactivation of chronic infection in severely immunocompromised mice. These findings provide proof of concept for the development of small molecule inhibitors of CDPK1 for treatment of CNS toxoplasmosis.

INTRODUCTION

Toxoplasma gondii is a widespread protozoan parasite of animals that causes zoonotic infections in humans. Although most human cases are well controlled, infection in immunocompromised patients leads to serious sequelae, including toxoplastic encephalitis and pneumonia, which are life-threatening if not treated.1 Although the advent of HAART therapy has reduced the frequency of toxoplasmosis as an opportunistic pathogen in developed countries, it is still a serious complication in many parts of the world where patients do not have adequate access to testing or treatment for HIV infection.2–5 Additionally, toxoplasmosis can cause serious problems in organ transplant and cancer chemotherapy patients due to their immunocompromised status.6 Furthermore, toxoplasmosis is a recognized cause of severe ocular disease in healthy adults in some locations such as Brazil.7

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Current therapy for toxoplasmosis is based on combination of pyrimethamine, which blocks dihydrofolate reductase (DHFR), and sulfadiazine, a folate antagonist. Collectively these drugs disrupt replication by inhibiting nucleic acid synthesis. This drug combination is efficacious in treating acute infection through blocking replication of tachyzoites. Unfortunately pyrimethamine is associated with several adverse side effects including anemia due to bone marrow suppression, and many patients experience allergic reactions to sulfonamide drugs. Chronic infections caused by *T. gondii* are typified by slow growing bradyzoites that reside within thick-walled tissue cysts. The emergence of bradyzoites upon cyst rupture is thought to give rise to daughter cysts that sustain the chronic infection and contribute to recurrence of actively replicating tachyzoites when the parasite reverts to the lytic form. Hence, treatments that could block re-emergence from the tissue cyst or block invasion of host cells by bradyzoites could interrupt this cycle and eliminate chronic infection. Unfortunately, current therapies that inhibit DHFR and antagonize the folate pathway are not effective at clearing chronic infection, as evidenced by the high relapse in immunocompromised patients when therapy is discontinued, presumably due to the slow and sporadic replication of bradyzoites.

One of the key steps in defining new leads for therapeutic intervention is to identify essential pathways that can be targeted by small molecules. One potential new target that fulfills these criteria is calcium dependent protein kinase 1 (CDPK1) in *T. gondii*. Genetic depletion or chemical inhibition of CDPK1 blocks adhesion secretion and compromises motility, cell invasion, and egress, thus demonstrating the essentiality of this target. The X-ray crystal structure of CDPK1 also revealed an unusual feature in the ATP-binding pocket where a glycine residue occupies the gatekeeper position where there is normally a larger hydrophobic residue. The presence of G gatekeeper is entirely unprecedented in human kinases, and CDPK1 is the only kinase in *T. gondii* with this unusual feature. As a consequence, CDPK1 is exquisitely sensitive to bulky ATP competitive inhibitors such as pyrazolopyrimidines (PP), which mimic the nucleotide binding interactions with the hinge region of human kinases. All compounds were synthesized using previously described methods to form the pyrazolopyrimidine scaffold via methylene or heteroatom linkages (X) in combination with t-butyl or various R₂ groups at the N1 position (Scheme 1). The hSrc kinase was chosen for initial counter-screening because it contains a threonine (T) gatekeeper residue that represents one of the smallest amino acids found in native human kinases. All compounds were synthesized via methylene or heteroatom linkages and tested for their ability to inhibit parasite growth in vitro using *T. gondii* lines expressing different heteroatom linkers to the core scaffold created by the G gatekeeper.

These features have been exploited to develop PP analogs that are potent inhibitors of CDPK1 in *T. gondii*, and the most promising of these compounds show good efficacy in mouse models of toxoplasmosis.

Our prior studies involving derivatives with substituents (R₁) to the C3 position of the pyrazolopyrimidine (PP) core revealed the importance of the methylene linkage at C3 to confer flexibility of R₁ substituent binding. However, compounds with the methylene linkage overall were not stable in the presence of rat liver microsomes, potentially due to the methylene bridge being subject to cytochrome P450 metabolism. To address these deficiencies, we have more fully explored the design and synthesis of different heteroatom linkages at the C3 position and expanded the diversity of substituents at the R₂ position. Collectively, these rationally driven modifications have identified new TgCDPK1 inhibitors with excellent potency in addition to improved selectivity, metabolic stability, and in vivo efficacy in controlling toxoplasmosis in the mouse.

## RESULTS

### Design and Synthesis of New PP Analogs Targeting CDPK1

To identify compounds with improved metabolic stability that would also exploit structural differences between CDPK1 and human Src (hSrc) active sites, we designed and synthesized a compound library containing varied functional groups at R₁ attached to the C3 position of the PP core (Scheme 1).

![Scheme 1](image)

These were either linked to the core pyrazolopyrimidine or pyrrolopyrimidine scaffold via methylene or heteroatom linkages (X) in combination with t-butyl or various R₂ groups at the N1 position (Scheme 1). The hSrc kinase was chosen for initial counter-screening because it contains a threonine (T) gatekeeper residue that represents one of the smallest amino acids found in native human kinases. All compounds were synthesized via previously described methods to form the pyrazolopyrimidine scaffold or with advanced pyrrolopyrimidine intermediates. Methods to generate compounds with methylene linkage at C3 have been previously reported, and heteroatom linkages were incorporated using established aryl coupling chemistry (Scheme 1). Detailed procedures for chemical synthesis and compound characterization are presented in the Supporting Information.

### Analysis of New PP Analogs Modified at the R₁ Position

To develop a structure–activity relationship (SAR) profile, we investigated the effects of modifying the R₁ group and different linkers to the core scaffold at position C3 in a series of compounds that contained tertiary butyl at the N1 position (Figure 1A). We tested compounds for their potency against CDPK1 in vitro, using an ELISA assay for phosphorylated CDPK1. We also observed increased metabolic stability of the ether, thioether, or amine linkage at C3 have been previously reported, and heteroatom linkages were incorporated using established aryl coupling chemistry. Finally, we screened each analog for stability in vitro in the presence of rat liver microsomes, as a surrogate for estimating in vivo metabolic stability.

Starting with parent compound 1, replacement of the C3 methylene linker with an ether, thioether, or amine linkage improved metabolic stability for ether and amine linkages (Figure 1A). This result confirms XenoSite in silico predictions of metabolism at the methylene as in compound 1, which is less likely with ether and amine linkages as in compounds 2 and 4 but is still possible with thioether linkages as in 3 (Supplementary Figure S1). The thioether linkage was also associated with substantial loss of activity in the parasite inhibition assay (Figure 1A). We also observed increased metabolic stability of the ether linkage over methylene for another compound set consisting of...
compounds 5 and 6. Analogs bearing halogen substitutions were generally more stable, especially in the presence of the ether linkage. The addition of some meta substituents on the benzyl ring was associated with loss of metabolic stability, despite having an ether linkage, for example, addition of the methoxy group in compound 9 and the methyl group for compound 10 (Figure 1A). Since the ether linkage generally improved metabolic stability while maintaining efficacy against parasite replication, we selected ether linkages for further optimization.

Structural Interactions between PP Compounds and CDPK1. To better understand the binding interactions of PP analogs and the CDPK1 enzyme, we solved a series of high-resolution cocystal X-ray structures, using previously described methods.14 The cocystal structure of CDPK1 with the lead compound 1 revealed a characteristic hydrogen-bonding (H-bonding) pattern of interaction between the aminopyridine and the kinase hinge. More specifically, the primary amine at C4 of the PP core acts as a H-bond donor in an interaction with the backbone carbonyl of glutamate 129, and the N5 in the PP core acts as a H-bond acceptor in an interaction with the backbone amide of tyrosine 131 (Figure 1B). Importantly, the cocystal structures of compounds 1–4 bound to CDPK1 show that these modifications do not substantially change the binding conformations (Figure 1C). Key H-bonding interactions between the PP core and the hinge region are maintained with R1 occupying the hydrophobic pocket formed by the presence of the small G gatekeeper residue (Figure 1C).

Analysis of New PP Analogs Modified at the R2 Position. Previous studies of PP analogs have shown that larger substituents are tolerated at the R2 position and can lead to gain of potency and selectivity for CDPK1.21 To explore the size tolerance within the CDPK1 pocket at this region, we generated a series of analogs with cyclic amines at R2 (Figure 2A). Structural studies have shown that methylpiperidine in the R2 is positioned to form a H-bond with glutamate 135 in the ribose binding region of the ATP pocket of CDPK1.19,20 A cocystal structure of compound 13 confirmed that this interaction is also present in the series studied here (Figure 2B). The piperidine is predicted to be protonated under physiological conditions, and as shown in the cocystal structure, the additional H likely forms a salt bridge with glutamine 135 (Figure 2B). We explored a series of cyclic amines based on piperidine where we elucidated the importance of proper placement and direction of the amine to form the favorable charge–charge salt bridge interaction with E135. We found that any deviation from the piperidine of 13 in this series abrogated CDPK1 activity and decreased inhibition of...
parasite replication in culture (Figure 2A). Compounds 16 and 15, which contain a tetrahydropyran and pyridine group, respectively, each significantly lost potency, emphasizing the key contribution of the H-bond donor of the piperidine (Figure 2A). On the other hand, all cyclic amines showed greatly enhanced stability in the rat liver microsome assay thus providing much support for further R3 modification as an approach to improving metabolic stability (Figure 2A).

In another series, we explored the combination of methylpiperidine at R2 together with the prior functional groups at R1 (Figure 3A). Consistent with findings above, the methylpiperidine containing analogs generally showed improved metabolic stability in comparison to their t-butyl counterparts (Figure 1A, 3A). For example, the presence of the methylpiperidine in compound 17 could impart stability despite having a methylene linkage to C3 of the PP core (Figure 3A). Compounds with additional halogens in the benzyl ring at R1 gained further increases in microsome stability (e.g., 21 and 22) (Figure 3A). Furthermore, there were no significant differences between pyrazolopyrimidine and pyrrolopyrimidine as the core scaffold as demonstrated by compounds 18 vs 23 (Figure 3A).

Despite the reproducible ability of methylpiperidine to improve microsome stability throughout the entire series, this modification also resulted in reduced biochemical potency and ability to block parasite replication. Hence, incorporation of methylpiperidine at R2 results in a trade-off between improved metabolic stability and loss in potency. This effect may be explained by the larger size of these inhibitors, which may not be as easily accommodated in the CDPK1 binding pocket. The combination of ether linked R1 as m-Cl with R2 piperidine as in compound 13 showed the largest improvement over parent compound 1, while retaining reasonable activity in both inhibition of CDPK1 and parasite growth (Figure 3A). In contrast, methylation of the piperidine nitrogen in compound 25 led to a loss in potency, further highlighting the importance of the H-bonding ability of the protonated amine (Figure 3A). Converting the piperidine to a 6-membered ring δ-lactam in compound 26 also lead to a loss in enzyme potency and a complete loss in antiparasitic activity (Figure 3A). We also explored modifying the piperidine by addition of difluorine (di-F), as such modifications are known to favorably affect the properties of drug-like molecules.32 The di-F modification in compound 24 vs 13, which both have ether-linked R3 substitutions to the PP core, slightly improved enzyme inhibition and antiparasitic activity (Figure 3A). A homology model of 24, based on the cocrystal structure of compound 13 with CDPK1, suggests that the piperidine amine is still in an appropriate position to interact with E135 (Figure 3B, Table 1). We also made a similar modification of di-F in the piperidine of compound 18 to generate the compound 28, both of which have a thioether linkage connecting R1 to C3 in the PP core. Surprisingly in this case, the di-F modification reduced enzyme activity and greatly diminished antiparasitic activity. Modification of R2 to contain a trifuoromethylcyclopropyl moiety was also synthesized in an attempt to reduce metabolic instability, based on a previous study showing this modification can improve stability of biaryl compounds relative to those containing t-butyl.33 This compound 27 lost potency (Figure 3), suggesting it does not optimally fit in the CDPK1 ATP binding pocket; moreover it did not improve stability, perhaps due to the methylene linkage of the trifuoromethylcyclopropyl moiety to N1 of the core.

Inhibition of CDPK1 in Vitro Correlates with Parasite Growth Inhibition. To compare the potency of all of the new PP analogs synthesized here, we plotted the log10 values for CDPK1 enzyme inhibition (IC_{50}) vs log10 values for parasite growth inhibition (EC_{50}) (Figure 4A). Fitting these data with a linear regression analysis indicated a reasonable correlation (r^2 = 0.58), supporting the conclusion that the activity of these compounds against parasite growth stems largely from inhibition of CDPK1. However, the fact that this correlation is not higher suggests that in vitro potency is also affected by other factors. Some inhibitors lie above the diagonal line, which represents a perfect correlation, indicating they are less potent in blocking parasite growth, perhaps due to differences in cell permeability or efflux (Figure 4A). Among the most potent inhibitors was the initial lead 1, and derivatives that contain an oxygen linkage at R1 (e.g., 2 and 10), as well as those that contain the methylpiperidine at R2 (e.g., 13, 24) (Figure 4A). Based on a combination of potency, selectivity, and PK properties, we prioritized compounds and selected these five analogs for further study. Other compounds
that were potent in both assays were deprioritized for lack of specificity (e.g., 4) or lack of stability (e.g., 9).

Metabolic Stability and Cellular Update of PP Analogs. To help choose PP analogs most likely to be active in vivo, we broadly examined the physical properties, metabolic stability in mouse liver microsomes, and cellular uptake of a select group of PP analogs (Table 1). All compounds have modest cLogP values, consistent with their intermediate hydrophobicity, and are predicted to have relatively small total polar surface area, properties that should facilitate penetration of the CNS (Table 1). Consistent with the findings in rat liver microsomes, the ether linkage in compound 2 reduced metabolic clearance in mouse liver microsomes when compared to compound 1, although both values are still relatively high (Table 1). Compound 10, which had similar clearance to compound 1 in rat liver microsomes despite having an ether linkage, was more rapidly cleared in mouse liver microsomes (Table 1). Stability was further improved when the R2 group was switched from t-butyl to methylpiperidine as in compounds 13 and 18. However, these latter two compounds, which contain a basic amine group that is charged at neutral pH, were also associated with substantial efflux in MDCK cells (Table 1). In addition to the many other useful aspects of fluorine substitutions in medicinal chemistry, in some contexts it has been shown to reduce the basicity of proximal amines by lowering the pKa.34 Accordingly, we modified the piperidine ring by addition of difluorine (di-F), a modification that reduced the pKa and nicely eliminated the efflux problem of compounds 13 and 18.

Table 1. Physical Parameters, Metabolic Stability, Efflux, And Toxicity of Select Compounds

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<tr>
<th>cmpd</th>
<th>ClogP</th>
<th>tPSA</th>
<th>pK_a</th>
<th>protein binding (%)</th>
<th>P_app 10^-6 cs/s A to B</th>
<th>P_app 10^-6 cs/s B to A</th>
<th>efflux ratio</th>
<th>microsomes</th>
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<td>0.9</td>
<td>0.887</td>
<td>&gt;10</td>
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“Determined in Chemdraw Ultra 12.0, PerkinElmer Informatics. "Apparent permeability (Papp), apical to basolateral (A-B). tPapp (B to A)/Papp (A to B). tCLint = k/P, where k is the elimination rate constant and P is the protein concentration in the assay. tInhibition of replication of human HFF cells.

Figure 3. Exploration of methylpiperidine at R2 for enhanced metabolic stability. (A) Structures of CDPK1 inhibitors highlighting modifications at R1 with methylpiperidine at R2 of a pyrazolopyrimidine core are shown in the top section, while modification of one compound to contain a pyrrolopyrimidine core is shown between the dotted lines. Structures of CDPK1 inhibitors highlighting modifications at R2 on a pyrazolopyrimidine core that were generated in attempts to reduce cellular efflux are shown in the bottom section of the table. Biochemical inhibition (IC50) of CDPK1, inhibition of parasite growth (EC50), off-target biochemical inhibition (IC50) of Src, metabolic stability in rat microsomes (half-life in minutes), and calculated clogP values. IC50 and EC50 values are the average of 2 or more biological replicates assays. (B) Homology model of compound 24 based on the cocrystral structure of compound 13 and CDPK1.
As such, we profiled our initial lead compound (Supplementary Table S2). Although compounds 13 and 24 exhibited IC_{50} values of $\leq$100 nM at 2 $\mu$M ATP, these values were increased almost 10-fold in the presence of 100 $\mu$M ATP (Supplementary Table S2). Additionally, we profiled an earlier inhibitor 3-methyl-benzyl-PP (compound 1 in ref 23) to compare our initial lead 1 in this prior series. We determined that the selectivity profile for both of these compounds was similar (Supplementary Figure S3), while selectivity was greatly increased for compounds 13 and 14. Full inhibitor profiling data is provided in the Supporting Information (Supplementary Tables S3–S5).

Potency of Select PP Analogs against ex Vivo Bradyzoites. Previous studies have emphasized the ability of PP analogs to block host cell invasion and egress by the rapidly growing tachyzoite stage of T. gondii. To examine the ability of our PP inhibitors to block invasion of bradyzoites, we purified mature tissue cysts from the brains of chronically infected mice and treated them in vitro with selected PP inhibitors. We compared the effects of treatment for short intervals (i.e., 4 h followed by wash-out and further culture in the absence of compound) vs continuous treatment during a 10–14 day plaking assay. Consistent with previous reports showing that treatment with pyrimethamine requires overnight treatment to be effective in blocking tachyzoite growth, we observed that short-term treatment with pyrimethamine had minimal effect on infection by bradyzoites, while continuous treatment blocked...
Figure 5. Human kinome profiling of compounds to determine inhibitor selectivity. Selectivity of compounds toward a panel of kinases in the ThermoFisher ACCESS program that are inhibited (red) by more than 50% or show greater than 50% tracer displacement (blue). Circle size reflects percent inhibition or displacement in the presence of inhibitor, and percentages are the mean of 2 data points. The % inhibition value for Src is shown as a yellow triangle and is emphasized by a dashed line connecting an * to the yellow triangle. Kinome tree images were generated using KinMap® and the kinase tree illustration is reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com). Inhibitors were screened at 1 μM.

Table 2. In Vivo Pharmacokinetic Properties Based on Plasma Levels of Select Compounds

| cmpd | route | dose, mg/kg | Tmax, h | C0/Cmax | AUCinf, ng/(mL h) | AUClast, ng/(mL h) | t1/2, h | CL, mL/(min kg) | Vss, L/kg | %F
|------|-------|-------------|---------|---------|-----------------|-----------------|---------|---------------|----------|-------
| 1    | iv    | 3           | 0.25    | 6892.94 | 1075.29         | 1081.76         | 2.06    | 46.22         | 1.09     | 26    |
| 2    | iv    | 3           | 0.25    | 5471.09 | 1146.49         | 1149.99         | 1.56    | 43.48         | 1.10     | 31    |
| 10   | iv    | 3           | 0.25    | 2741.80 | 619.33          | 625.04          | 1.74    | 80            | 2.32     | 4     |
| 13   | iv    | 3           | 0.25    | 76.69   | 85.47           | 91.42           | 91.42   | 94            | 1.3      | 94    |
| 24   | iv    | 3           | 0.25    | 1205.19 | 975.90          | 988.58          | 1.56    | 50.58         | 3.68     | 1.3  |

*Cmax for po route, C0 (initial concn). CL, clearance. Vss, steady-state volume of distribution. %F, Oral availability determined from AUCinf.

plaque formation (Figure 4B). In contrast, the PP inhibitors showed good (e.g., 13), or excellent (e.g., 1, 24), ability to block infection following even short-term incubation (Figure 4B). Because the compounds were only present during the initial interaction of the parasite with the host cell monolayer, we interpret these results to indicate that PP inhibitors can block bradyzoite entry into host cells during the 4 h treatment period, resulting in greatly diminished plaque formation. Additionally, continual culture with PP compounds resulted in greater inhibition (Figure 4B), consistent with its ability to block the multiple rounds of invasion and egress that occur during plaque formation, although it is likely that tachyzoites predominate during these longer-term assays.

**Mouse Pharmacokinetic Studies.** We examined the pharmacokinetics (PK) of several PP inhibitors in mice following either iv injection or po administration in mice. Compounds 1 and 2 showed reasonable oral bioavailability (F) ranging from ~25% to 30%, Cmax levels, and moderate clearance (CL) values that were below the level of single pass circulation through the liver in mouse (∼90 mL/(min·kg)) (Table 2). Compound 10 showed a higher clearance rate but had similar half-life (T1/2) to 1 and 2 of ~1.5–2 h (Table 2). However, this compound suffered from poor oral bioavailability (Table 2). Although compound 13 had greater in vitro stability in microsomes (Table 1), it also displayed rapid clearance in vivo, perhaps due to efflux as indicated above. Compound 13 also showed lower Cmax values and high tissue exposure as evidenced by its volume of distribution (Vss) (Table 2). Finally, compound 24 showed the best combination of properties with good Cmax following oral dosing, moderate clearance rate, T1/2 ≈ 1.5 h, and very high oral availability (e.g., ~94%) (Table 2). These collective properties of compound 24 provided the best PK profile and highest values for AUC, reflecting prolonged serum exposure following oral dosing.

Based on these favorable properties, we examined the PK of 24 in mice using increasing oral doses from 10, 25, and 50 mg/kg (Figure 4C). At higher dose, 24 showed a two-phase decay curve in plasma, with rapid initial drop followed by a more gradual plateau that was seen at 25 and 50 mg/kg (Figure 4C). Based on its protein binding properties (12% free in serum), the concentration of compound 24 in serum would need to be 2.08 μM, for the free value to attain a level equivalent to the in vitro EC50 of ~0.250 μM. This corresponds to a value of 856 ng/mL in serum, as shown by the red line in Figure 4C. The protein bound fraction in brain was slightly higher at 97.2%, or 2.8% free, and the ratio of total compound in the CNS vs serum was 5.6, for an effective brain vs plasma ratio ~1.3. Consequently, we would expect that the free concentration of compound 24 in the brain was approximately the same or slightly higher than that shown for serum in Figure 4C.
Treatment of Acute Toxoplasmosis. Based on its favorable PK parameters, we tested compound 24 for its ability to prevent toxoplasmosis in the mouse model. Following an initial loading dose of 50 mg/kg, 24 was administered orally at 25 mg/kg BID for 6 days, beginning 24 h after challenge with tachyzoites of the ME49 strain of *T. gondii*. Treatment with 24 decreased the frequency of lethal infection (Figure 6A), and this was associated with reduced weight loss (Figure 6B) and with decreased tissue burdens of the parasite, as revealed by bioluminescent imaging (Figure 6C). Consistent with a decrease in acute burden, there were fewer tissue cysts formed in the brains of chronically infected mice following treatment with 24 (Figure 6D). The dramatic ability of 24 to decrease dissemination was further shown by bioluminescent imaging, where control animals showed extensive expansion of the parasite with in the peritoneum at day 8 and spread to the head at day 18 (Figure 6E,F). In contrast, treated mice were able to restrict the growth of the parasite to the site of injection and did not show detectable signal in the head (Figure 6E,F). Following treatment with 24, many animals showed tissue cyst levels below the levels of detection (40 cysts per brain) (Figure 6D). These animals were subsequently bioassayed by oral feeding of 20% of their brain homogenate into a naive interferon gamma receptor knockout mouse (*Ifngr1−/−*). Although 5 of 6 recipient animals succumbed to infection, reflecting a low level of chronic infection in the donor, one remained alive, indicating that compound 24 treatment had resulted in a complete cure in this animal (Figure 6D, red triangle).

Treatment of Reactivated CNS Toxoplasmosis. To determine whether compound 24 could prevent reactivation of chronic toxoplasmosis, we utilized mice lacking the IFNγ receptor (i.e., *Ifngr1−/−*), which are highly susceptible and rapidly succumb to infection. Treatment of such animals with sulfadiazine will suppress the growth of tachyzoites, while
allowing the development of chronic tissue cysts. Subsequent removal of sulfadiazine results in rapid reactivation, characterized by CNS encephalitis, pneumonia, and death. We injected Ifngr1−/− mice with a type II strain capable of causing chronic infection and treated them from day 2 to 22 with sulfadiazine in the drinking water, which prevented otherwise lethal infection. Following removal of sulfadiazine (48 h later), mice were treated with compound 24 at a dose of 25 or 40 mg/kg BID or with vehicle only control by oral gavage for 8 days (Figure 7A). Withdrawal of sulfadiazine from the control animals lead to rapid reactivation of chronic infection and led to death of the control mice within 10 days (Figure 7B). In contrast, treatment with compound 24 prolonged survival (Figure 7B). We also monitored the progression of infection using bioluminescence. At the beginning of the treatment period, all of the animals were negative for bioluminescence, consistent with the infection being chronic. Monitoring of the control animals revealed that reactivation was first centered in the CNS, with later spread to the lungs prior to death (Figure 7C). In contrast to the rapid expansion of parasites seen in control mice, the bioluminescence signal was muted in compound 24 treated mice, and reactivation was significantly delayed (Figure 7C). Consistent with this delay in reactivation, compound 24 treated mice survived significantly longer (Figure 7B). One animal treated with compound 24 at 25 mg/kg and two animals treated at 40 mg/kg remained negative for bioluminescence signals and survived for an additional 22 days after removal of compound (red triangles in Figure 7B). Subsequent bioassay of brain homogenates from these treated mice into naïve Ifngr1−/− mice did not result in transfer of infection, confirming that these animals had been cured (Figure 7B).

### DISCUSSION

In previous work, we demonstrated that PP inhibitors containing meta chloro benzyl substituents at C3 of the PP core provide potent inhibition of CDPK1 in T. gondii. However, when coupled with isopropyl or t-buty1 at R2, these compounds suffer from metabolic instability. Our profiling of host kinases here also reveals that compounds like 1 lack specificity, and this is also likely true of other related derivatives that share this basic architecture. To alleviate this problem, we altered the linkage connecting R1 to C3 from a methylene to a heteroatom consisting of O, S, or N. An ether linkage proved most optimal in increasing stability and selectivity, while compounds with a thioether linkage were typically less active and compounds with an amine linkage were less selective. The reasons for these differences are not exactly clear as cocrystal structures of these analogs showed very close overlap of the analogs in the binding pocket. The lower activity of the thioether linkage may relate to increased stability and selectivity, while compounds with a thioether linkage were typically less active and compounds with an amine linkage were less selective. The reasons for these differences are not exactly clear as cocrystal structures of these analogs showed very close overlap of the analogs in the binding pocket. The lower activity of the thioether linkage may relate to increased stability and selectivity, while compounds with a thioether linkage were typically less active and compounds with an amine linkage were less selective. The reasons for these differences are not exactly clear as cocrystal structures of these analogs showed very close overlap of the analogs in the binding pocket. The lower activity of the thioether linkage may relate to increased stability and selectivity, while compounds with a thioether linkage were typically less active and compounds with an amine linkage were less selective. The reasons for these differences are not exactly clear as cocrystal structures of these analogs showed very close overlap of the analogs in the binding pocket. The lower activity of the thioether linkage may relate to increased stability and selectivity, while compounds with a thioether linkage were typically less active and compounds with an amine linkage were less selective. The reasons for these differences are not exactly clear as cocrystal structures of these analogs showed very close overlap of the analogs in the binding pocket. The lower activity of the thioether linkage may relate to increased stability and selectivity, while compounds with a thioether linkage were typically less active and compounds with an amine linkage were less selective.
scaffold described here also resulted in dramatic improvement in specificity over host kinases compared to the initial lead compounds 1 and 2. Compounds 13 and 24 only potently inhibited a single human kinase, CK1ε, under conditions tested here. Surprisingly, CK1ε is also sensitive to other PP analogs including 1-NM-PP1 and 1-NA-PP1, despite its methionine gatekeeper, which is normally a hallmark for resistance to such analogs.39 The sensitivity of CKε to PP analogs has been attributed to an unusual flexibility in the M side-chain that allows access of bulky PP analogs into the ATP-binding pocket.40 pck retains good potency against parasite growth in a cell-based assay, while computational predictions of the compound dissemination to the CNS. Indeed, treatment was sufficient to prolong survival of such severely immunocompromised mice, but treated animals ultimately always succumb to infection following removal of these drugs. Similarly, treatment with sulfadiazine is only able to prevent lethal infection in this model, but neither eliminate the tissue cysts, such that when it is removed, animals quickly succumb due to reactivation of rapidly growing tachyzoites. Treatment with compound 24 prolonged survival of Ifng1−/− mice and resulted in a radical cure in some animals, a result not previously seen with other compounds. The efficacy of compound 24 in this model likely stems from its ability to block tachyzoite invasion and egress, but it was also able to block bradyzoite invasion in vitro, suggesting it may directly affect the formation and turnover of cysts in vivo. Other PP analogs have been reported to reduce the chronic cyst burden in infected mice, albeit at very high serum doses, which exceed the level where they are selective for CDPK1.19 In contrast, compound 24 retains antitoxoplasma activity at very modest serum levels, which are well within the range where it is selective for CDPK1 in T. gondii.12,23

**CONCLUSIONS**

CDPK1 is an essential kinase in T. gondii and hence has been the focus of development of potent and selective inhibitors. Here we report new PP inhibitors that were designed to improve metabolic stability and specificity for CDPK1. The optimal molecule in this series, compound 24, showed low nanomolar inhibition of CDPK1 in vitro, submicromolar inhibition of parasite growth in vitro, and improved metabolic stability. In vivo administration of this analog, which showed excellent oral availability and moderate PK parameters, decreased the severity of acute infection, reduced tissue cyst levels, and delayed reactivation of chronic toxoplasmosis in the mouse. Additionally, compound 24 is remarkable in being able to completely cure a portion of immunocompromised animals. As radical cure is currently not possible for humans infected with T. gondii, compounds that can achieve this goal with higher efficacy would greatly enhance the potential utility of PP analogs for treatment of toxoplasmosis. Collectively, these advances underscore the utility of the PP scaffold as a new therapeutic agent for treatment of acute and chronic toxoplasmosis.

**EXPERIMENTAL SECTION**

Chemical Synthesis. Starting materials, solvents, and reagents obtained commercially were reagent grade and were used without further purification. NMR spectra were obtained at the University of California, San Francisco, NMR facility: 1H NMR were recorded on a Bruker AvanceIII HD 400 at 400 MHz, and 13C spectra were recorded on a Bruker AvanceIII HD 400 at 400 MHz or a Bruker Advance DRX500. High-resolution mass spectra (HRMS) were acquired by electrospray ionization (ESI) in positive ion mode using Finnigan LTQ FT mass spectrometer (Thermo) at the QB3 Chemistry Mass Spectrometry Facility (University of California, Berkeley). Samples were directly injected into ESI source via syringe pump with flow rate 5 μL/min. Reactions were monitored by thin layer chromatography (TLC), using Merck silica gel 60 F254 glass plates (0.25 mm thick). Flash chromatography was conducted with Teledyne Isco RediSep Rf silica flash cartridges on a Teledyne Isco CombiFlash Rf+. All RP-HPLC were performed with a Waters 2545 binary gradient module equipped with an XBridge prep C18 column using H2O + 0.1% formic acid and CH3CN + 0.1% formic acid (5−98% gradient) while monitoring at 254 nm. All final compounds were ≥95% pure as measured by liquid chromatography−mass spectrometry (LCMS) using a Waters Acquity UPLC/ESI-TQD BEH C18 (1.7 μm) column using H2O + 0.1% formic acid and CH3CN + 0.1% formic acid (5−95% gradient) over 1.8 min at
600 μL/min. Full compound characterization details are presented in the Supporting Information. All compounds reported here were screened for Pan Assay Interference (PAINS) and found to pass all the filters contained in the PAF-Drug4 Web server (http://fafdrug4.mit.univ-paris-diderot.fr/).

**Parasite Strains and Cell Lines.** Tachyzoites of *T. gondii* strains were grown in monolayers of human foreskin fibroblasts (HHF) maintained in complete medium (DMEM containing 10% FBS, 10 mM glutamine, and 10 μg/mL gentamycin) at 37 °C in 5% CO₂. Following natural egress, tachyzoites were purified in HBSS containing 10 mM HEPES, 0.1 mM EGTA, and separated from host debris using 3.0 micro polycarbonate membrane filters, followed by centrifugation at 400g. HFF monolayers obtained from an anonymous donor were provided by Dr. John Boothroyd’s laboratory (Stanford University). All strains and host cell lines were determined to be mycoplasma negative using the e-Myco plus kit (Intron Biotechnology).

**Enzyme Expression and Purification.** Full-length *T. gondii* CDPK1 was expressed with a C-terminal His tag in E. coli that produces the LamP phosphatase. Following overnight growth in E. coli, soluble proteins were purified using NdeI and XhoI restriction enzymes as described previously. CDPK1 was expressed in BL21 (DE3)-host cell lines were determined to be mycoplasma negative using the Dr. John Boothroyd (mti.univ-paris-diderot.fr/). Kinase reactions were conducted at 37 °C for 10 min using the Enzyme Kinase assay reagents as detailed in the Typical Procedure. The kinase reactions were conducted using the type I RH strain, 2F clone, which expresses bacterial β-galactosidase (β-gal), as described previously. Diffraction crystals were obtained by cocryocrystallization with compounds 3 (PDB code: 2SW8), 1 (PDB code: 4H88), 13 (PDB code: 4W80), and 4 (PDB code: 2W91). Crystallographic data were collected at beamline 19ID of Argonne National Laboratory’s Advanced Photon Source (http://www.sbc.anl.gov/index.html) and processed using HKL3000. All structures were solved using Phaser for molecular replacement and the previously deposited PDB files were used as search models. The models were refined using BUSTER and REFMAC combined with iterative manual model building using the molecular graphics program Coot. The geometry of all final models was checked using MolProbity. Crystallographic details and refinement statistics are summarized in Table S1. Atomic coordinates and experimental data will be released on publication.

**Homology Modeling.** Compound 24 was modeled using the co-crystal structure of compound 13 (PDB code: 2SW8) in Molecular Operating Environment (MOE) version 2015.1 by Chemical Computing Group. Dihydroxyamine was added to the piperidine of compound 13 using the ligand builder to create compound 24, and the structure was prepared and minimized using LigX with the Amber10:EHT force field (used Protonate3D for protonation, allowed ASN/GLN/HIS “Flips” in Protonate3D, deleted water molecules farther than 4.5 Å from compound 24 or CDPK1; tethering parameters: receptor strength at 5000 to minimize large changes to the CDPK1 backbone, fixed atoms farther than 8 Å away from compound 24, hydrogens close to compound 24 not fixed, and refined compound 24—CDPK complex to an RMS gradient of 0.1 kcal/mol Å). Enzyme Assays and IC₅₀ Values. CDPK1 activity was monitored based on phosphorylation of tyrosine-2 peptide (Callibiochem), which was detected using mAb MS-66E (MBL, Intl, Corp) using an ELISA protocol described previously. Kinase reactions were conducted at 30 °C for 20 min in 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 2.3 mM CaCl₂, 0.1 mM EGTA, 0.005% Tween 20. Reactions were conducted using 25 μM ATP (the Kₘ for the enzyme) using 140 ng of kinase per reaction. To determine the potency of inhibitors, duplicate wells were treated with a range of compound concentrations from 10 μM to 0.5 nM. Inhibitors were preincubated with the enzyme in the reaction buffer for 10 min before addition of ATP. Individual IC₅₀ values were determined from two or more independent biological replicates and are reported as mean ± SD. For compounds that showed >50% inhibition at 10 μM, serial dilutions from 10 μM to 0.5 nM were tested in duplicate to derive IC₅₀ values.

**Kinase Screen.** Human kinase profiling was conducted using the ThermoFisher Scientific SelectScreen ACCESS program to profile 489 kinases utilizing three assay formats: Z′-LYTE (284 kinases), AdaptaSelectScreen (39 kinases), and LanthaScreen (166 kinases). Z′-LYTE and AdaptaSelectScreen yield % inhibition values based on the mean of 2 points that measure the ability of the compound to inhibit an active kinase. LanthaScreen yields % displacement values based on the mean of 2 points that measure the ability of the inhibitor to displace a tracer bound to an inactive or weakly active kinase. Inhibitors were tested at 1 μM. Parasite Growth Assays and EC₅₀ Values. Parasite growth inhibition assays were conducted using the type I RH strain, 2F clone, which expresses bacterial β-galactosidase (β-gal), as described previously. Compounds dissolved in DMSO at 10 mM stocks were diluted in medium to two times final concentrations and added to an equal volume of medium containing 5 × 10⁶ parasites and incubated for 20 min. Mixtures of compounds (ranging from 10 μM to 0.01 nM) containing 0.1% (v/v) DMSO or DMSO alone was added to monolayers of HFF cells grown in 96 well plates, centrifuged at 300g for 5 min, and returned to culture at 37 °C, 5% CO₂. After 4 h, the plates were washed to remove extracellular parasites and compounds and then returned to culture for 72 h. At the end of the incubation period, the monolayer was lysed in 1% Triton X-100 and β-gal activity monitored using 1 mM chloro phenol red-β-D-galactopyranoside by absorption at 570 nm, as described previously. Individual EC₅₀ values were determined from three or more independent biological replicates and are reported as mean ± SD.
As a positive control, mitomycin C (10 μM) was included in each plate. Compounds were tested in duplicate, in two biological replicates, and values are reported as means ± SD.

**In Vitro Analysis of Uptake, Efflux, Metabolism, and Protein Binding.** Assays to monitor uptake and efflux of compounds by polarized epithelial cells were conducted by Absorption Systems Inc. (Exton, PA, USA). In brief, Caco-2 cells (Clone C2BBe1 from ATCC) were grown to confluence on collagen-coated polycarbonate membranes on transwell plates. Permeability assays were conducted in Hank’s balanced salt solution, containing 10 mM HEPES, 10 mM glucose, pH 7.4. Compounds were added at 5 μM to the apical (A) vs basolateral (B) side, and duplicate samples were taken at 120 min. Samples were analyzed by LC-MS/MS and expressed as apparent permeability (P_app) for uptake (A to B) vs efflux (B to A) and efflux ratio (P_app(B to A)/P_app(A to B)).

The stability of compounds in the presence of rat or mouse liver microsomes was conducted by Absorption Systems Inc. In brief, rat liver microsomes or mouse rat liver microsome were incubated with compounds at 1 μM in a shaking water bath for 60 min. Aliquots were withdrawn at intervals, extracted, and analyzed by LC-MS/MS to evaluate the remaining parent compound. Half-lives were calculated using a single-phase exponential decay equation (GraphPad). Intrinsic clearance was calculated as C_Lin = k/e, where k is the elimination rate constant and P is the protein concentration in the assay. For plasma protein binding, compounds were incubated at 5 μM in CD-1 mouse plasma, and protein bound fraction was determined by equilibrium dialysis.

**In Vivo Pharmacokinetic Studies.** Pharmacokinetic (PK) studies were conducted by Sai Life Sciences Limited (India). Compounds were administered orally in 50% PEG-400 in PBS, while for iv administration, compounds were dissolved in PBS and administered by iv injection. The constant treatment plate was allowed to incubate for 10 min at 37 °C. NaCl, 60 mM HCl, 0.1 mg/mL pepsin (1:1000 activity), incubated for 10 min at 37 °C, and then neutralized by addition of sodium carbonate solution (94 mM). Liberated bradyzoites were treated with 1% sodium dodecyl sulfate (SDS) and 1% Triton X-100 and the DNA was stained with 0.1% crystal violet stain, and plaques were tracked over the first 30 days. At the end of 30 days, tissue cysts in the brain were quantified by staining with Dolichos biflorus lectin that was labeled with FITC, and microscopic examination, as described previously. For samples where no cysts were observed (lower threshold = 40 per brain), residual infection was tested by bioassay into a naive recipient. For bioassay experiments, 200 μL of brain homogenate was injected ip into a recipient ifngr1−/− mouse, which lacks the ability to control parasite proliferation and readily succumbs to infection.

**Treatment during Reactivation of Chronic Toxoplasmosis.** To provide a model for treatment during reactivation of chronic infection, we used a modification of a previously published protocol. 50 51 Male and female Ifngr1−/− mice were orally infected with 5 cysts of the type II ME49Δfl::FLUC line, obtained from the brains of chronically infected wild type CD-1 mice. Animals were treated with sulfadiazine (0.25 g/L in the drinking water) from day 2 to 22. Two days (48 h) after removal of the sulfadiazine, compound 24 formulated in 25% PEG-400–PBS containing 5% DMSO or 25% PEG-400–PBS−5% DMSO alone (control) was administered by oral gavage for a total of 8 days. One group of treated mice received on day 1 an initial loading dose of compound 24 at 50 mg/kg in the morning, followed 12 h later by a second dose of 25 mg/kg, and then 25 mg/kg BID for 7 additional days. Bioluminescence imaging (see below) was used to monitor infection from day 6 to 20, and weight loss and survival were tracked over the first 30 days. At the end of 30 days, tissue cysts in the brain were quantified by staining with Dolichos biflorus lectin that was labeled with FITC, and microscopic examination, as described previously. 50

**Bioluminescence Imaging.** Animals were monitored for bioluminescence using a Xenogen IVIS200 instrument, and images were obtained using the Xenogen Living Image software (Caliper Life Sciences). Animals were anesthetized using 2% isoflurane and injected ip with 0.15 μL of 1 mL of 1x naïve recipient ifngr1−/− mice, as described above. There were no differences in outcome associated with the sex of animals.
Compound 24 homology model (PDB)
Molecular formula strings (CSV)

Accession Codes
The structure factors and pdb coordinates have been deposited at the protein databank (PDB) with the coordinates 4HFP, 5W8R, 5W80, 5W9E, and 5W91. Compound 24 was modeled using the co-crystal structure of compound 13 (PDB code: 5W9E). Authors will release the atomic coordinates and experimental data upon article publication.

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS USED:

ANOVA, analysis of variance; β-gal, β-galactosidase; BID, bis in die (twice a day); CDPK1, calcium dependent protein kinase 1; CNS, central nervous system; EC50, 50% effective concentration; ELISA, enzyme-linked immunosorbent assay; HAART, highly active antiretroviral therapy; HFF, human foreskin fibroblast; HIV, human immunodeficiency virus; IC50, 50% inhibitor concentration; FLUC, firefly luciferase; SAR, structure–activity relationship

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